Br. J. Pharmac. (1975), 55, 431-437

TOLERANCE TO MORPHINE-INDUCED CALCIUM DEPLETION IN REGIONAL BRAIN AREAS: CHARACTERIZATION WITH RESERPINE AND PROTEIN SYNTHESIS INHIBITORS

D.H. ROSS

Departments of Pharmacology and Psychiatry, The University of Texas, Health Science Center at San Antonio, 7703 Floyd Curl Drive, San Antonio, Texas 78284, U.S.A.

1 Administration of a single dose of morphine sulphate (25 mg/kg) induces tolerance to calcium depletion lasting seven days.

2 There are no apparent changes in calcium content in any of eight discrete brain regions throughout this seven day period.

3 Pretreatment with reserpine (5 mg/kg) did not alter the ability of morphine to induce tolerance. Reserpine alone produced no tolerance to its own calcium depleting action.

4 Cycloheximide (500 μg/kg) but not chloramphenicol (200 mg/kg) effectively prevented development of tolerance.

5 It is concluded that the induction of tolerance to calcium depletion seen after morphine may involve changes in various proteins in membranes of synaptic origin.

Introduction

Requirement for calcium in membrane stabilization and function of neuronal systems is well established (Katz & Miledi, 1965; Triggle, 1972), particularly in excitation-coupling mechanisms (Somlyo & Somlyo, 1968; Douglas, 1968), and in the regulation of hormone-receptor interactions (Rasmussen, Goodman & Tenenhouse, 1972), but it has been only briefly studied in relation to mechanism of action of opiates. Kakunaga, Haneto & Hano (1966) reported reversal of morphine analgesia by calcium ions. Morphine has been found to alter calcium binding to phospholipids and gangliosides *in vitro* (Mule, 1969; Greenberg, Diecke & Long, 1972) and opiate ligand binding to isolated membrane fractions has been reduced by the addition of physiological concentrations of calcium (Pert & Snyder, 1973a,b; Hitzeman, Hitzeman & Loh, 1974).

Recent studies in this laboratory have been concerned with the *in vivo* effects of morphine on regional brain calcium (Ross, Medina & Cardenas, 1974). Morphine has been shown to decrease calcium in regional brain areas of the rat in a uniform fashion (Cardenas & Ross, 1975). This depletion was antagonized by naloxone, mimicked by levorphanol, but not by dextrophan. By the use of two non-analgesic central nervous system (CNS) depressants, reserpine and pentobarbitone, to characterize the morphine effect, evidence for more than one calcium pool in the CNS was obtained.

To establish an effect as directly related to opiate action, criteria such as those outlined above must be satisfied. In addition, a major criterion fulfilled in previous investigations has been the development of tolerance to the particular parameter being examined. In continuing an investigation into the role of calcium in the actions of morphine on the CNS, this study demonstrates the development of tolerance to the morphine-induced calcium depletion. Further, this tolerance is characterized by a calcium and amine depleting agent, reserpine, and protein synthesis inhibitor, cycloheximide. The results suggest that synthesis of membrane protein may be involved in the rapid induction of tolerance to the calcium depleting effect.

Methods

Preparation of tissues

Male Sprague-Dawley rats weighing 175-250 g were used in all experiments. Tissues were prepared from animals receiving saline (0.9% w/v NaCl solution) or the appropriate drug in saline solution

according to methods previously described by Cardenas & Ross (1975). Tissues were prepared for calcium analysis by removing samples of eight anatomically discrete brain regions as outlined by Glowinski & Iversen (1966). The tissues were prepared from animals receiving saline or the appropriate drug in the equivalent volumes. Animals were killed at stated times; the brain was rapidly removed and rinsed in ice cold saline. Suitable amounts of tissue (10-25 mg) were removed and placed in preweighed disposable test tubes (Corning, diSPo, 16 x 100 mm culture tubes) together with 200 μl of concentrated HNO_3 (analytical grade) and the entire contents evaporated to a dry white ash over a hot plate. Any incomplete ashing as evidenced by dark brown flecks was treated with a second 200 μl volume of acid and the ashing procedure repeated. The residue was allowed to cool for 15 min then resuspended in a solution of 0.5 ml of 0.1 N HCl + 4.5 ml of 1% lanthanum (as the oxide in 0.1 N HCl). This solution was then read for calcium at the appropriate instrument settings.

Analysis of calcium

The concentration of calcium in regional areas of the rat brain was determined with the aid of a Perkin-Elmer atomic absorption spectrophotometer Model 403. The wavelength was set at 212 nm for calcium with a slit setting of 4, with the remainder of the instrument pre-adjusted in accord with the Perkin-Elmer Analytical Manual, 1973. A combination calcium-magnesium-zinc lamp was used with a current of 20 milliamperes. External standards were prepared by first diluting the calcium stock solution with 0.1 N HCl. Appropriate working standards were then prepared by further dilution in 1% lanthanum (as the oxide) in 0.01 N HCl. Lanthanum is included to prevent interference from high concentrations of phosphate normally found in brain tissue (Hanig & Aprison, 1967).

Materials

Drugs used in this study and their sources were as follows: morphine sulphate (Eli Lilly Laboratories, Indianapolis, Indiana), reserpine (Serpasil Brand, Ciba Geigy, Summit, New Jersey), cycloheximide (Sigma Chemical Corporation, St. Louis, New Jersey) and chloramphenicol (Chloramycetin, Park Davis & Company, Detroit, Michigan). Lanthanum and calcium standards were obtained from Research Chemical Corporation, Sun Valley, California, and Fisher Scientific Company, Fairlawn, New Jersey, respectively.

Statistics

Group and paired comparisons were made using Student's *t*-test as previously outlined by Snedecor & Cochran (1969).

Results

Time course of tolerance to a single dose of morphine

Our previous studies have demonstrated that calcium content in brain regions is maximally depressed 30 min after administration of morphine (Cardenas & Ross, 1975). After a single dose of morphine the average depletion throughout eight brain regions is approximately 35%. Calcium values returned to control levels within the first 24 hours. No changes were observed from this point throughout the next seven days when samples were studied at three and seven days.

To satisfy the criteria of tolerance to morphine as a reduced responsiveness to its repeated administration, the experiments outlined in Table 1 were performed. Each of three groups of animals received an induction dose of morphine (25 mg/kg) at zero time, and subsequently, another 25 mg/kg, 30 min before they were killed on days 1, 3 or 7. It can be concluded that, after a single induction dose, a second dose of morphine has no significant effect on brain calcium at days 1 and 3 since the regional values are not significantly different from the corresponding ones in rats treated with saline alone (Table 1) but are significantly different from those in rats given only one dose of morphine for 30 min (see footnote to Table 1). In contrast, calcium levels on day 7 in animals receiving an induction dose of morphine are lower than at days 1 or 3 although they are not decreased by as much as has been shown previously to occur 30 min after the administration of a single dose of morphine (Cardenas & Ross, 1975).

Effects of reserpine on calcium content

Reserpine was employed in an attempt to define more closely possible relationships between catecholamines and tolerance to the calcium-depleting effect of morphine. Previous studies (Cardenas & Ross, 1975) have demonstrated that values of the calcium content are reduced 2 h after the administration of reserpine (5 mg/kg) but return to normal at 24 hours.

Three groups of animals were used. One group received saline alone (Table 2, column 1). The second group received reserpine (5 mg/kg)

followed 24 h later by one dose of morphine (25 mg/kg); the animals were killed 30 min after injection of morphine. It can be seen (Table 2, column 2) that prior treatment with reserpine had no significant effect on the ability of morphine to cause a decrease in calcium content in these animals. The third group was used to evaluate the ability of reserpine to induce tolerance to its own calcium-depleting action. When reserpine was given as an induction dose of 5 mg/kg, followed after 24 h by a second dose (5 mg/kg), the calcium values were depleted (at 2 h) by the second dose. A comparison of these values may be made with the values of calcium 2 h after a single treatment with reserpine (5 mg/kg) (see footnote, Table 2); there is no significant difference.

Effects of reserpine on development of tolerance

Reserpine was also evaluated for its ability to alter tolerance. Table 3 depicts the effects of reserpine on tolerance to calcium depletion in eight brain regions. Reserpine was administered as a 2 h pretreatment followed by the tolerance regimen as

Table 1 Acute tolerance to calcium depleting effect of a single dose of morphine* in different brain regions of the rat

	Tissue calcium, μg/g wet wt. (mean ± s.e. mean)†			
Brain region	*Day 1*	*Day 3*	*Day 7*	*Saline-treated*
Hypothalamus	53.2 ± 0.5	57.0 ± 5.0	54.3 ± 4.7	55.8 ± 2.0
Hippocampus	55.3 ± 2.9	60.6 ± 3.3	44.6 ± 6.7‡	59.0 ± 2.1
Corpus striatum	57.2 ± 1.3	51.6 ± 1.2	48.6 ± 5.4	56.6 ± 1.9
Cortex	56.8 ± 1.4	57.4 ± 1.8	54.3 ± 3.8	52.1 ± 0.7
Cerebellum	54.2 ± 2.2	47.3 ± 4.6	39.3 ± 1.2‡	54.8 ± 0.8
Medulla pons	52.2 ± 4.3	46.0 ± 3.2	43.0 ± 3.7‡	54.8 ± 0.9
Midbrain	59.0 ± 4.6	54.0 ± 3.2	53.6 ± 3.7	56.0 ± 0.9
Thalamus	48.0 ± 2.1	51.0 ± 1.8	42.0 ± 3.5‡	54.1 ± 1.1

All values expressed are the mean of separate determinations from 8-12 animals.
* Morphine sulphate tolerance regimen was performed as follows: morphine (25 mg/kg i.p.) was given at zero time. Animals received a second dose 30 min before they were killed 1, 3 and 7 days later; animals were killed 30 min after the second morphine dose (25 mg/kg). Acute treatment with morphine (25 mg/kg) (30 min) has been previously shown to cause an average depletion of 35% throughout eight regions (Cardenas & Ross, 1975).
† Values for day 1 and day 3 are not significantly different from saline-treated rats.
‡ Values for day 7 represent transition from tolerance to non-tolerance state and are significant at $P < 0.01$.

Table 2 Effects of reserpine on morphine depletion of calcium in different regions of rat brain

	Tissue calcium, μg/g wet wt. (mean ± s.e. mean)		
Brain regions	*Control*	*Reserpine**	*Reserpine†*
Hypothalamus	55.8 ± 2.0	37.5 ± 4.1	40.8 ± 3.0
Hippocampus	59.0 ± 2.1	30.3 ± 2.5	37.5 ± 2.0
Corpus striatum	56.6 ± 1.9	33.3 ± 1.4	40.3 ± 2.5
Cortex	52.1 ± 0.7	33.5 ± 3.1	40.6 ± 0.9
Cerebellum	54.8 ± 0.8	38.8 ± 2.6	34.8 ± 2.8
Medulla pons	54.8 ± 0.8	39.4 ± 3.3	36.2 ± 2.2
Midbrain	56.0 ± 0.9	34.5 ± 3.6	41.2 ± 2.6
Thalamus	54.1 ± 1.1	37.2 ± 2.2	38.7 ± 2.1

Values are mean of separate determinations from 10-12 animals.
* Reserpine (5 mg/kg) given 24 h before morphine (25 mg/kg). Animals killed 30 min after morphine. All values significantly different from controls ($P = 0.001$). Animals given reserpine (5 mg/kg) followed 24 h later by saline did not have values significantly different from those of saline controls.
† Tolerance to reserpine was evaluated by giving reserpine (5 mg/kg) at zero time followed by same dose 24 h later. Animals were killed 2 h after the second dose and calcium determined. All values are significantly different from controls. It has previously been shown that reserpine (5 mg/kg) causes an average calcium depletion of 33-36% throughout eight brain areas at 2 h (Cardenas & Ross, 1975).

outlined in Table 1. Only small decreases in brain calcium content were observed for day 1, 3 or 7 after reserpine pretreatment. The levels are not significantly different over all from those listed in Table 1 for days 1, 3 and 7 respectively.

Effects of protein synthesis inhibition on calcium content and development of tolerance

Cycloheximide (500 μg/kg, i.p.) or chloramphenicol (200 mg/kg, i.p.) were administered in a single dose to three groups of rats. One hour later, the tolerance schedule of injection of morphine was started as outlined in Table 1; Table 4 illustrates the results of these experiments. Calcium values after cycloheximide were significantly depleted at all three days compared to controls receiving appropriate solutions of saline instead of morphine. However, chloramphenicol proved ineffective in altering the development of tolerance at day 1, 3 and 7. Both cycloheximide and chloramphenicol alone produced no changes in control values for calcium content. Cycloheximide did not alter the ED_{50} for calcium-depletion by morphine (Ross, unpublished

Table 3 Effects of reserpine* on development of tolerance to calcium-depleting effect of morphine

	Tissue calcium, μg/wet wt. (mean ± s.e. mean)		
Brain region	*Day 1*	*Day 3*	*Day 7*
Hypothalamus	53.6 ± 1.4	51.1 ± 2.7	42.6 ± 3.9
Hippocampus	51.2 ± 3.3	55.2 ± 1.7	51.6 ± 2.0
Corpus striatum	49.3 ± 1.2	52.1 ± 2.4	46.3 ± 4.4
Cortex	49.3 ± 1.2	52.1 ± 2.4	46.3 ± 4.4
Cerebellum	48.5 ± 4.1	46.8 ± 0.8	52.3 ± 3.0
Medulla pons	57.3 ± 2.7	45.7 ± 4.6	55.3 ± 2.3
Midbrain	46.6 ± 2.0	55.6 ± 1.6	56.0 ± 3.0
Thalamus	47.3 ± 5.6	54.1 ± 1.4	48.0 ± 4.0

Values represent mean of 8-10 animals.
* Reserpine (5 mg/kg) was administered as 2 h pretreatment followed by the morphine tolerance regimen outlined in Table 1. It has previously been demonstrated that reserpine alone (5 mg/kg) produced an average depletion of 33-36% in eight discrete brain regions (Cardenas & Ross, 1975).
Day 1, 3 or 7 significantly different from reserpine controls.

Table 4 Effects of cycloheximide* (Cyclo) and chloramphenicol† (Chlor) on development of tolerance to morphine in regional brain areas

	Tissue calcium, μg/g wet wt. (mean ± s.e. mean)						
		Day 1		*Day 3*		*Day 7*	
Brain region	*Control*	*Cyclo*	*Chlor*	*Cyclo*	*Chlor*	*Cyclo*	*Chlor*
Hypothalamus	55.8 ± 2.0	32.9 ± 2.9	55.9 ± 2.0	32.5 ± 2.6	54.6 ± 0.9	26.6 ± 0.3	51.7 ± 0.8
Hippocampus	59.0 ± 2.1	36.5 ± 4.2	57.0 ± 1.4	34.5 ± 2.5	55.3 ± 1.4	30.3 ± 1.8	52.5 ± 2.0
Corpus striatum	56.6 ± 1.9	34.0 ± 2.7	53.8 ± 2.1	34.7 ± 2.9	56.0 ± 1.0	25.6 ± 3.5	53.6 ± 0.8
Cortex	52.1 ± 0.7	30.7 ± 4.1	57.6 ± 3.6	35.7 ± 4.5	54.0 ± 1.6	32.3 ± 2.9	51.3 ± 1.1
Cerebellum	54.8 ± 0.8	31.6 ± 3.7	56.0 ± 2.8	31.0 ± 2.5	53.3 ± 1.6	26.5 ± 2.4	53.7 ± 0.8
Medulla pons	54.6 ± 1.5	33.3 ± 1.8	56.4 ± 1.1	31.3 ± 5.0	52.0 ± 0.5	33.3 ± 6.6	52.2 ± 2.3
Midbrain	56.0 ± 0.9	31.0 ± 0.9	57.0 ± 2.0	31.5 ± 2.6	56.2 ± 1.8	23.3 ± 2.6	50.5 ± 2.0

Values represent mean of separate determinations from 6-8 animals.
* Cycloheximide (500 μg/kg) or (†) chloramphenicol (200 mg/kg) administered 1 h before start of tolerance regimen for morphine.
No significant differences for any of the three days or any of the individual brain regions when compared to controls. Corpus striatum, cerebellum and thalamus were significantly lower after cycloheximide on the seventh day ($P = 0.01$).

observation) an observation which has also been made by Tulunay & Takemori (1974).

Discussion

Administration of a single dose of morphine sulphate (25 mg/kg) induces tolerance to depletion of brain calcium for periods up to seven days. Throughout this period, there were no significant changes in any of the regional brain areas. Acute tolerance to a second dose of morphine persisted three days after the first exposure of the animal to the drug. By the seventh day, however, tolerance to the second dose of morphine was beginning to decline. Tolerance to single doses of opiates over short time periods has been previously reported. For example, Smith, Karmin & Gavitt (1966) administered levorphanol in single doses to mice and found tolerance to the cataractogenic effect of a second dose. This dose was administered within a period of five days. Cox, Ginsburg & Osman (1968) reported tolerance to analgesia as early as 4 h after the beginning of 5-10 mg kg^{-1} h^{-1} constant infusion.

Many attempts have been made to link changes in tissue levels and turnover rates of certain neurotransmitters with the development of tolerance to various actions of opiates. Theoretical possibilities for tolerance mechanisms involving cellular adaptation have also been based on alteration of levels of and sensitivity to various neurochemical mediators. Reserpine, which has been previously shown to alter brain calcium levels (Raduco-Thomas, 1971; Cardenas & Ross, 1975), did little to prevent the development of tolerance to the morphine-induced depletion of calcium. This lack of effect of reserpine in preventing tolerance may not be surprising. It has been previously demonstrated that at least two pools of calcium exist, one morphine-sensitive and protected by naloxone, the other reserpine-sensitive but not protected by naloxone (Ross *et al.*, 1974; Cardenas & Ross, 1975). The calcium depleted by reserpine would conceivably leave the morphine-sensitive pool intact. Supporting this idea of distinct pools of calcium is the observation that while reserpine causes calcium depletion, it does not produce tolerance to its own depleting effect (Table 2) suggesting that some particular structural requirement of the membrane may be necessary for activating tolerance to calcium depletion. Secondly, treatment with reserpine 24 h before a single dose of morphine did not alter the ability of morphine to deplete calcium (Table 2), nor the development of tolerance (Table 3). These observations would also suggest that optimum endogenous levels of biogenic amines may not be required for morphine to induce depletion of calcium.

In contrast to the lack of effect of reserpine on the development of tolerance, pretreatment with the protein synthesis inhibitor cycloheximide produces a significant blockade of tolerance development for periods lasting up to seven days (Table 4). The mechanism of action for cycloheximide has been reported as the direct prevention of ribosomal movement along the m-RNA chains (Wettstein, Noll & Penman, 1964; Colombo, Felicetti & Boglioni, 1965). Therefore, it might be reasoned that the induction of tolerance to the depletion of calcium may in some way be connected to the translational aspect of protein synthesis. In support of this reasoning, Datta & Antopol (1973) have demonstrated in mice that chronic opiate treatment produces a decrease in aminocyl synthetase activity that is both dose and time-dependent.

In contrast to the effects seen after cycloheximide treatment, chloramphenicol (200 mg/kg) had no effect on development of tolerance to calcium depletion when administered as a single dose prior to initiation of tolerance. Previous investigations have shown that cycloheximide as well as other inhibitors of protein synthesis effectively abolish tolerance initiated by opiate drugs (Cohen, Keats, Krivoy & Ungar, 1965; Loh, Shen & Way, 1969; Cox & Osman, 1970; Feinberg & Cochin, 1972). Our data confirm these observations with cycloheximide and also support the lack of effects of chloramphenicol on tolerance development previously reported by Cox & Osman (1970). The inability of chloramphenicol to suppress the development of tolerance significantly may be explained in the following manner. Although the drug is reported to alter mammalian protein synthesis (Wheeldon & Lehninger, 1966), it has been previously demonstrated that chlroamphenicol does not alter protein synthesis in cerebral tissue in doses five time greater than that required to alter liver synthesis activity (Gordon & Deanin, 1968). The lack of effect may also be related to its action at mitochondrial protein synthesis, rather than protein synthesis of synaptic membrane origin, as suggested by Morgan & Austin (1968). In support of this idea, Barondes (1974) has recently reported studies indicating about 80% of protein synthesis by synaptosome fractions is inhibited by cycloheximide while about 20% is sensitive to chloramphenicol and is thought to be of mitochondrial origin.

Data presented in this paper demonstrate that tolerance to morphine may be induced by a single dose and persist for periods of up to seven days Cycloheximide acted effectively to prevent

induction of tolerance while chloramphenicol was without activity. Reserpine, previously shown to interrupt analgesia tolerance, was also without effect. Based on the presumed site of action of cycloheximide, it is suggested that rapid changes in protein synthesis may take place within the synaptic membrane. Tolerance to morphine may involve rapid changes in the calcium-protein associations of the synaptic membranes.

This research was supported in part by NIDA-01168 and Pharmaceutical Manufacturers Association Research Grant.

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(Received June 18 1975. Revised August 4 1975.)